Exhibit B

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Molecular Cloning and Characterization of Senescence-Related Genes from Carnation Flower Petals[1]

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ABSTRACT

The senescence of carnation (*Dianthus caryophyllus* L.) flower petals is associated with increased production of ethylene which plays an important role in regulating this developmental event. Three senescence-related cDNA clones were isolated from a cDNA library prepared from mRNA isolated from senescing petals. These cDNAs are representative of two classes of mRNAs which increase in abundance in senescing petal tissue. The mRNA for one class is present at low levels during the early stages of development and begins to accumulate in mature petals prior to the increase in ethylene production. The accumulation of this mRNA is reduced, but not eliminated, in petals treated with aminooxyacetic acid, an inhibitor of ethylene biosynthesis, or silver thiosulfate, an ethylene action inhibitor. In contrast, expression of the second class of mRNAs appears to be highly regulated by ethylene. These mRNAs are not detectable prior to the rise in ethylene production and increase in abundance in parallel with the ethylene climacteric. Furthermore, expression of these mRNAs is significantly inhibited by both aminooxyacetic acid and silver thiosulfate. Expression of these mRNAs in vegetative and floral organs was limited to floral tissue, and predominantly to senescing petals.

Senescence represents the final stage in the development of a whole plant, organ, tissue, or cell. Flower petals are often the plant organ with the shortest life span, and as such provide a useful tissue for studying the mechanisms underlying control of senescence. It is clear that petal senescence is a highly controlled developmental event, which plays an important role in the overall reproductive development of the plant (2). In many species, petals function in the attraction of insects for pollination. Once pollination has occurred, and the function of the petals is complete, the metabolic resources of the petals are rapidly mobilized to the developing ovary leading to petal senescence. In the flowers of carnation (*Dianthus caryophyllus* L.), pollination induces senescence by signaling the petals to produce the phytohormone ethylene (18). In the absence of pollination, petal senescence still occurs and is associated with a climacteric-like increase in ethylene production (2, 16). It is clear that the increase in ethylene production plays a critical role in the coordination and regulation of petal senescence in carnation. Treatment of flowers with inhibitors of ethylene biosynthesis (4) or action (4, 22, 23) prevents typical petal senescence. Furthermore, exposure of presenescent flowers to ethylene hastens the onset of petal senescence.

The senescence of flower petals is associated with a series of physiological and biochemical changes. These include an increase in hydrolytic enzymes (1, 9), degradation of macromolecules (1, 14), increased respiratory activity (17), and a loss of cellular compartmentalization (8, 15, 20). Many of these processes are highly regulated and are the result of active metabolism (1, 2, 20). Analysis of total proteins isolated from carnation petals during the development of senescence revealed both increases and decreases in the levels of several polypeptides (25). *In vitro* translation of mRNAs isolated from carnation petals at various stages of development has shown that senescence is accompanied by changes in mRNA populations (25). Taken together, these results indicate that the development of petal senescence is associated with regulated changes in gene expression.

There is growing evidence that responses of plants to ethylene can include changes in gene expression (3, 6, 11, 12, 19, 26). In climacteric fruits such as avocado and tomato, ethylene has been shown to modulate the expression of several ripening-specific genes (5, 12). In addition, ethylene has been reported to increase the steady state levels of several mRNAs for enzymes involved in plant defense responses including chitinase (3), 4-coumarate:CoA ligase (6), and L-phenylalanine ammonia-lyase (6). We recently reported (26) that exposure of presenescent carnation petals to ethylene leads to premature petal senescence and induces changes in gene expression. Furthermore, most of the ethylene-induced mRNAs are similar to those which accumulate during natural senescence (25), based on the mol wt of their *in vitro* translation products.

We are interested in understanding how senescence in plants is controlled and have chosen to study the regulation of carnation petal senescence. In this organ, senescence is rapid and predictable and is regulated by the phytohormone ethylene. Analysis of petal senescence should serve as a model for senescence of other plant tissues and is an interesting system for studying the action of a specific hormone at the molecular level. Here we report the identification of several cDNA clones which have been used to examine the expression of senescence-related mRNAs during flower petal senescence and in response to ethylene.

MATERIALS AND METHODS

Plant Material

Carnation (*Dianthus caryophyllus* L. cv 'White Sim') flowers were harvested at anthesis from plants grown under green-

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house conditions and held in the laboratory as described (25). Vegetative and reproductive tissues were collected, immediately frozen in liquid nitrogen, and stored at −70°C until used for RNA isolation.

Chemicals

L-[^{35}S]Methionine at 1200 Ci/mmol and α[^{32}P]dCTP at >3000 Ci/mmol were obtained from New England Nuclear. Restriction enzymes were purchased from Promega Biotec and Bethesda Research Laboratories. Nick translation kit was obtained from Amersham. Chemicals used in cDNA synthesis were purchased from Bethesda Research Laboratories. λgt10 and Gigapack phage packaging extracts were purchased from Stratagene. All other chemicals and reagents were from Sigma Chemical Company.

RNA Extraction and Poly(A$^+$) Isolation

RNA was extracted from frozen tissue and poly(A$^+$) RNA isolated as described elsewhere (25, 26).

Construction and Screening of a cDNA Library

Double-stranded cDNA was synthesized from poly(A$^+$) RNA isolated from senescing carnation petals (6 d after harvest) essentially as described by Gubler and Hoffman (7). Following methylation, ligation of EcoRI linkers and digestion with EcoRI, cDNA molecules larger than 0.5 kb[2] were ligated to EcoRI digested λgt10 (10). The recombinant DNA molecules were packaged *in vitro* and used to infect *Escherichia coli* strain C600 hfl$^+$. Approximately 5 × 10^5 recombinant phage were present in this cDNA library. The library was screened by differential hybridization (10) using ^{32}P-labeled single-stranded cDNA, prepared from preclimacteric (day of harvest) and climacteric (6 d after harvest) petal poly(A$^+$) RNAs. Briefly, a portion of the library was plated at a density of 500 pfu/9 cm × 9 cm plate. Duplicate nitrocellulose lifts were taken from each plate, denatured, neutralized, and baked for 2 h at 80°C under vacuum (10). Each replicate lift was prehybridized for 4 h at 68°C in a solution containing 6 × SSC (1 × SSC is 0.15 M $NaCl_2$, 15 mM Na-citrate, pH 7.0), 0.1% SDS, 5 × Denhardt's mixture (1 × Denhardt's is 0.02% PVP, 0.02% Ficoll, 0.02% BSA), and 100 μg/mL denatured salmon sperm DNA. Hybridization was carried out for 18 h at 68°C in identical buffer containing 5 × 10^5 cpm/mL ^{32}P-labeled cDNA. Filters were washed at 68°C for 4 h in 4 changes of 3 × SSC, 0.1% SDS (w/v), dried, and exposed to Kodak XAR-5 film with an intensifying screen at −70°C for 18 h. A number of recombinant phage that hybridized more intensely with climacteric cDNA were picked and plaque purified through several rounds of screening. After purification, the cDNA inserts of these phage were isolated and subcloned into the EcoRI site of pUC18. Phage and plasmid DNAs were isolated and purified as described by Maniatis *et al.* (13).

[2] Abbreviations: kb, kilobase pair; AOA, aminooxyacetic acid; NBD, 2,5-norbornadiene; STS, silverthiosulfate; pfu, plaque-forming unit.

Northern and Slot Blot Hybridizations

RNA was electrophoretically separated on 1.2% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose. The nitrocellulose filter was prehybridized 4 h at 42°C in a solution containing 50% (v/v) formamide, 5 × Denhardt's, 0.1% SDS, 6 × SSPE (1 × SSPE is 0.15 M mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), and 100 μg/mL denatured salmon sperm DNA. Hybridization was carried out in identical buffer solution plus 5 × 10^5 cpm/mL denatured ^{32}P-labeled DNA probe for 18 h at 42°C. Plasmid DNA was labeled by nick translation to a specific activity of 2 × 10^8 cpm/μg DNA. The filters were washed in 3 × SSC, 0.1% SDS (w/v) for 4 h at 42°C, exposed to Kodak XAR-5 film with an intensifying screen at −70°C for 18 to 36 h. Total RNA was slot blotted according to manufacturers instructions using a Schleicher and Schuell Minifold II Slot Blot apparatus. Prehybridization and hybridization were carried out as described above for RNA-blot analysis.

Hybrid-Select Translation

mRNA corresponding to the cDNA clones was selected according to Maniatis *et al.* (13). Briefly, 10 μg of linearized plasmid DNAs from selected clones were denatured in 0.5 M NaOH, neutralized with the addition of hybrid select translation buffer (1 M NaCl, 0.3 M Na-citrate, 0.5 M Tris [pH 8]), and spotted onto 1 cm^2 nitrocellulose discs. The discs were air-dried and baked at 80°C under vacuum. Prehybridization was performed for 2 h at 42°C in 0.6 M NaCl, 20 mM Pipes, 2 mM EDTA, 0.1% SDS (w/v), and 100 μg/mL tRNA. The hybridization solution was the same as prehybridization except for the addition of 10 μg poly(A$^+$) RNA from d 6 petals. Total hybridization volume was 100 μL. The filters were hybridized for 18 h at 42°C. Following hybridization, filters were washed 10 times in 1 mL of buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 0.5% (w/v) SDS at 65°C, followed by two final washes in the same buffer without SDS. Bound mRNA was released into sterile H_2O by heating to 100°C for 2 min. The hybrid-selected mRNA was precipitated overnight at 20°C by the addition of 5 μg carrier tRNA, 1/20 volume of 4 M Na-acetate, and 2 volumes ethanol. Hybrid-selected mRNA was translated *in vitro* using rabbit reticulocyte lysate, separated by SDS-PAGE, and visualized by fluorography as previously described (25).

RESULTS

Ethylene Production and Carnation Petal Senescence

Ethylene production by carnation flowers and visible morphological changes exhibited by the petals during senescence are shown in Figure 1. The increase in ethylene represents the 'climacteric' phase of development and has been shown to be associated with increased respiratory activity in carnation petals (2, 17). In addition, carnation petals exhibit specific morphological changes which can be associated with ethylene production. For the first 4 d after harvest, ethylene production remained low (<1 nL/g fresh weight·h^{-1}) and the flowers showed no visible signs of senescence. An increase in ethylene production was evident by the fifth d after harvest and peaked

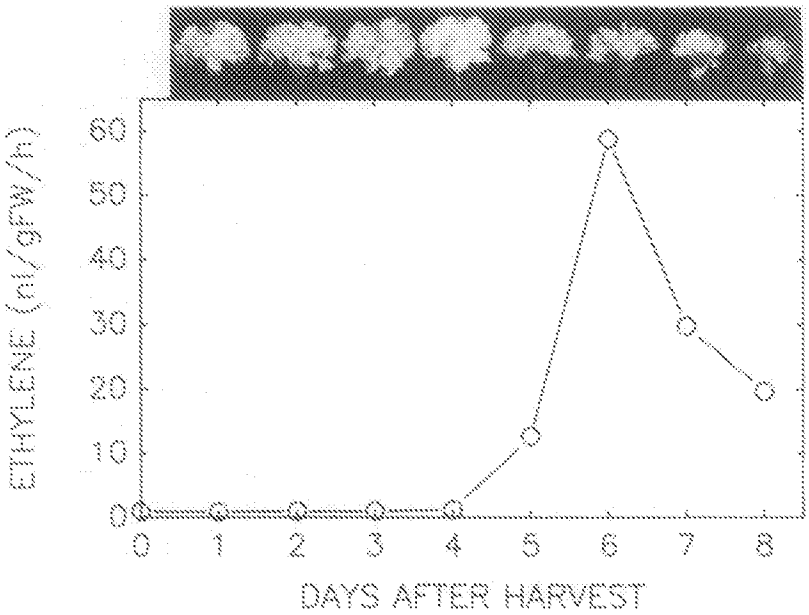

Figure 1. Ethylene production rate and morphological symptoms of carnation flower petal senescence. Individual flowers were placed in 1-L containers that were sealed for 30 min. A 1-mL sample was removed from the container atmosphere, and the ethylene content determined by gas chromatography (27). Petals have been shown to account for 80 to 90% of the total ethylene production by the whole flower (2).

on the sixth d when petals showed in-rolling and slight wilting. Ethylene production declined on d 7 and petals were severely wilted.

Isolation of Senescence-Related cDNA Clones

In order to isolate cDNA clones for genes exhibiting increased expression during petal senescence, a cDNA library was prepared from poly(A^+) RNA isolated from petals 6 d after flower harvest. Day 6 petals were used because at this stage ethylene production is maximal and visible symptoms of senescence are apparent (Fig. 1). It is clear that this is a critical stage in the coordination of biochemical events of petal senescence (2, 4, 15, 17, 25). Furthermore, recent results indicate that autocatalytic ethylene continues to play a role in the regulation of petal senescence until very late in the process since treatment of climacteric petals with the ethylene action inhibitor NBD results in reversal of senescence symptoms (24). We used a differential screening approach (10) to identify and isolate clones for genes exhibiting increased expression during petal senescence. Approximately 3000 clones were screened with ^{32}P-labeled cDNA from d 0 petals and d 6 petals. This primary screen identified 43 clones with enhanced expression in d 6 petals as compared to d 0 petals. These positive clones were replated at lower density (100 pfu/9 cm × 9 cm plate) and differentially screened. Additional screens revealed 15 clones hybridized more intensely to ^{32}P-cDNA from d 6 petals. In an attempt to determine if any of these clones were homologous, phage DNA was isolated and digested with EcoRI. We were unable to isolate cDNA inserts from 4 of the 15 individual clones, most likely as a result of modification to the EcoRI restriction sites during cloning. DNA homology hybridizations between the remaining clones indicated the cDNAs represented three gene families (data not shown). The cDNA represented by pSR5 was homologous with five other clones, while pSR12 was homologous to three additional clones. One clone, designated pSR8, was represented by a single cDNA clone. In addition, we isolated a clone, designated 3P, for use as a constitutively expressed control.

To confirm that the selected cDNAs represent mRNAs which increase in abundance during senescence, poly(A^+) RNA isolated from presenescent (d 0) and senescent (d 6) petals was separated on a denaturing agarose gel, blotted to nitrocellulose, and probed with nick translated plasmids. Each clone hybridized to a single size of mRNA and, with the exception of 3P, showed increased hybridization with mRNA isolated from senescing petals (Fig. 2). These results confirm the cDNAs represent mRNAs which increase in abundance in senescing petal tissue.

In Vitro Translation of Petal mRNAs and Hybrid-Select mRNAs

In an attempt to identify translation products of the cloned cDNAs, plasmid DNA was used to hybrid select mRNA from a population of messages isolated from d 6 petals. The cDNA clone pSR5 hybrid selected a transcript that directed the synthesis of an 81 kD polypeptide upon *in vitro* translation (Fig. 3). *In vitro* translation of mRNA from senescing petals revealed several new mRNAs, one of which yielded an 81 kD translation product, as previously reported (25). No translation products could be selected by pSR8 and pSR12. This may be a result of their relative low abundance or limited methionine residues, in which case the relative amount of radioactive label incorporation would be reduced.

Expression of Senescence-Related mRNAs during Petal Senescence

The expression of these mRNAs during the course of petal senescence was examined by hybridizing specific cDNA probes to RNA isolated from petals at various times after harvest (Fig. 4). The mRNA for pSR5 began to accumulate in petals by d 4, which preceded the increase in ethylene production and visible symptoms of senescence. In contrast,

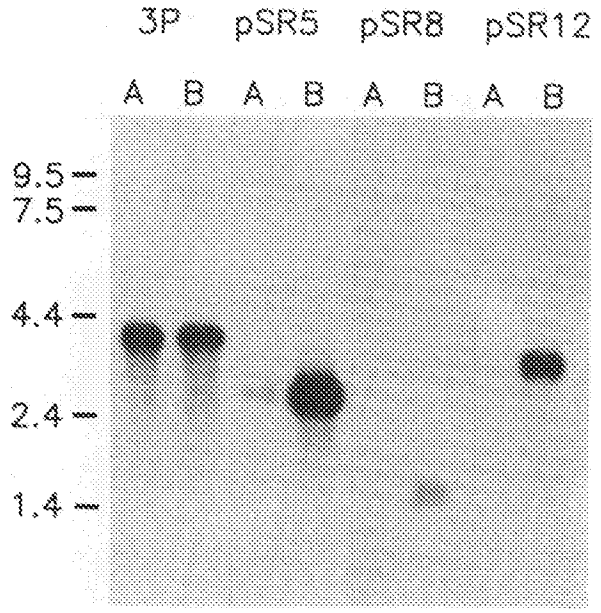

Figure 2. RNA blot analysis of mRNAs represented by various senescence-related cDNA clones. Poly(A$^+$) RNA (1.0 μg) was electrophoretically separated under denaturing conditions on 1.2% agarose, blotted, and hybridized to the ^{32}P-labeled DNA designated at the top of each pair of lanes. The poly(A$^+$) RNA was isolated from petals on the day of harvest (A) or 6 d after harvest (B). RNA standards (BRL) were run with the petal poly(A$^+$) RNA and the sizes are illustrated in kb. The blot was exposed to x-ray film with an intensifying screen at −70°C for 12 h.

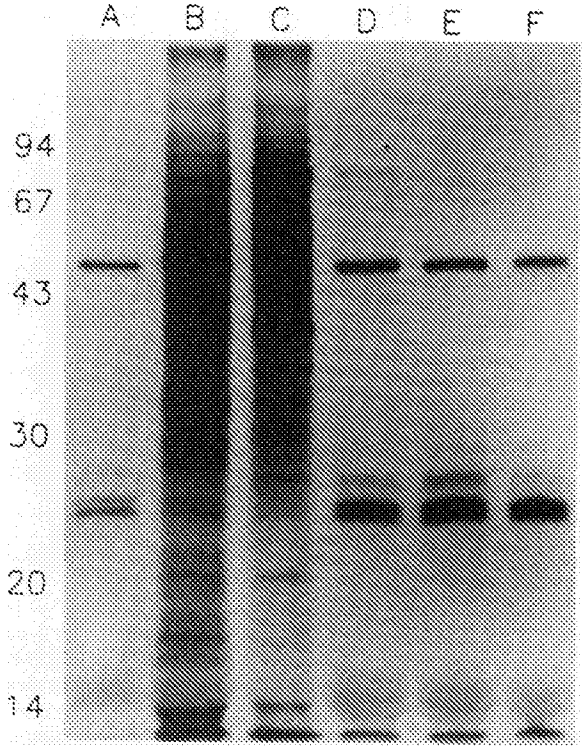

Figure 3. *In vitro* translation of total mRNAs from preclimacteric and climacteric carnation petals and mRNAs hybrid-selected using specific cDNA clones. Poly(A$^+$) RNA was isolated from carnation petals at day of harvest and 6 d after harvest. Specific mRNAs were purified from the d 6 poly(A$^+$) RNA population by hybridization with cDNAs bound to nitrocellulose (13). RNAs were translated *in vitro* using rabbit reticulocyte lysate, and subjected to SDS-PAGE as previously described (25). Lane A, no mRNA was added; lane B, d 0 poly(A$^+$) RNA; lane C, d 6 poly(A$^+$) RNA; lane D, clone pSR5 hybrid select mRNA; lane E, clone pSR8 hybrid select mRNA; and lane F, clone pSR12 hybrid select mRNA. Mol wt standards are in kD.

the mRNAs represented by pSR8 and pSR12 increased coincident with ethylene production beginning 5 d after harvest. In all cases, the mRNAs for senescence-related genes were most abundant in senescing petals. The mRNA for clone 3P did not change significantly in abundance during petal senescence.

Effects of Inhibitors of Ethylene Biosynthesis and Action on Expression of Senescence-Related mRNAs

The concomitant increase in abundance of mRNAs for pSR8 and pSR12 with the ethylene climacteric suggests that ethylene may play a role in their expression. In contrast, the accumulation of mRNA represented by pSR5 prior to the increase in ethylene indicates that the expression of this mRNA may be regulated by temporal factors. To determine the role of ethylene in the expression of these senescence-related mRNAs, flowers were treated on the day of harvest with AOA and STS, which are inhibitors of ethylene biosynthesis and action, respectively. Six d following the initiation of treatments, flowers treated with either agent showed no symptoms of petal senescence and had ethylene production rates of less the 1 nL/g fresh weight/h. In contrast, untreated flowers were exhibiting symptoms of senescence (petal in-rolling) and had entered the ethylene climacteric. Treatment of flowers with either AOA or STS only slightly reduced the accumulation of the mRNA represented by pSR5 (Fig. 5). In contrast, these treatments essentially prevented the accumulation of mRNAs represented by pSR8 and pSR12. These results show that increased ethylene biosynthesis and action (perception) are required for accumulation of mRNAs for pSR8 and 12, while other age related factors may play a role in the expression of pSR5.

Effects of Exogenous Ethylene on Expression of Senescence-Related mRNAs

Exogenous ethylene is capable of inducing premature senescence and many of the mRNAs that accumulate during this process (26). We therefore examined the effect of ethylene on the expression of these specific senescence-related mRNAs in young presenescent flowers. Flowers were harvested at anthesis and exposed to 7.5 μL/L ethylene for various durations after which RNA was isolated from petals and hybridized with labeled cDNA clones. The levels of senescence-related mRNAs in ethylene treated petals are shown in Figure 6. Within 3 h after the initiation of ethylene treatment, the level of each of the mRNAs represented by pSR5, pSR8, and pSR12 had increased significantly over the control. The accumulation of these mRNAs preceded any visible symptoms of petal senescence. An ethylene exposure of at least 6 h was required before in-rolling of petals, autocatalytic ethylene

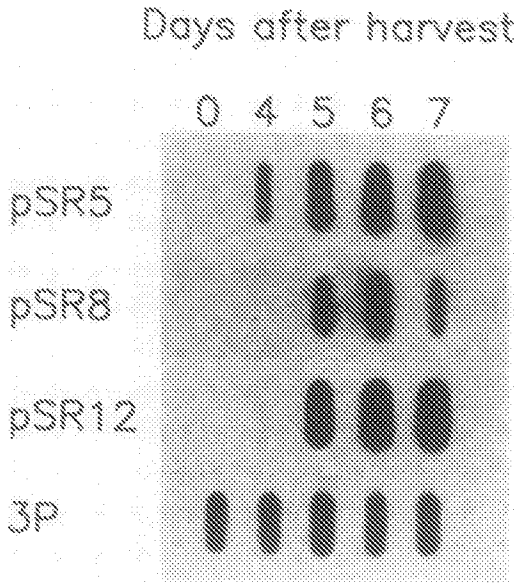

Figure 4. Accumulation of specific mRNAs during carnation petal senescence. Ten μg of total RNA isolated from petals at various times after harvest was slot-blotted onto nitrocellulose, and hybridized with ^{32}P-labeled cDNA clones. Petals exhibited initial morphological symptoms of senescence and increased ethylene production 5 d after harvest. Blots of pSR5, pSR8, and pSR12 were exposed to x-ray film with an intensifying screen at −70°C for 18, 36, or 18 h, respectively.

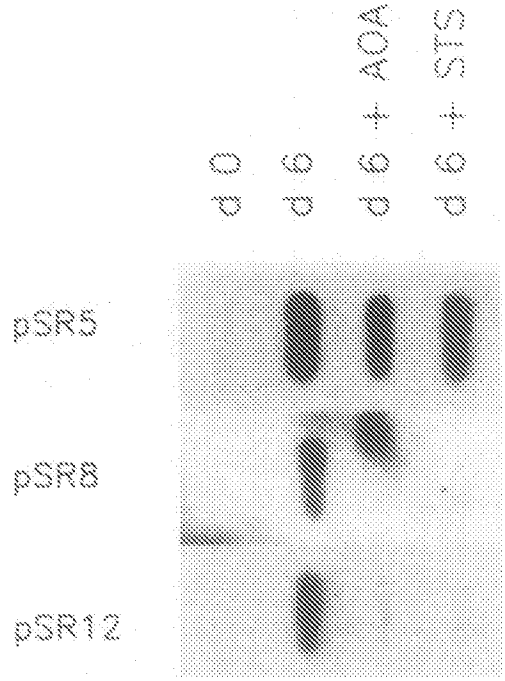

Figure 5. Effect of inhibitors of ethylene biosynthesis and action on the accumulation of specific mRNAs during carnation petal aging. Flowers were harvested on d 0 (d 0) and treated with either 4 mM AOA or 4 mM STS. STS was prepared as previously described (26). Following treatment with STS for 1 h, flowers were transferred to H_2O and held for 6 d. Flowers were treated continuously with AOA. Ten μg of total RNA isolated from petals the day of harvest (d 0) or 6 d after harvest (d 6) was slot-blotted onto nitrocellulose and hybridized with ^{32}P-labeled cDNA clones. Blots of pSR5, pSR8, and pSR12 were exposed to x-ray film with an intensifying screen at −70°C for 18, 36, or 18 h, respectively.

production, and premature senescence were observed (data not shown). The mRNA identified by 3P did not change in abundance during 12 h of ethylene treatment.

Organ Specificity of Senescence-Related mRNA Expression

We determined the organs in which these mRNAs were expressed by analyzing their abundance in various vegetative and floral organs. Total RNA was extracted from leaves, stems, roots, and floral organs and subjected to Northern blot analysis. As shown in Figure 7 none of the mRNAs represented by these cDNAs are detected in mature vegetative organs, even upon prolonged exposure of the film (data not shown). The senescence-related clones show increased hybridization in tissue from climacteric flowers, although the maximum accumulation of these mRNAs is in petals.

DISCUSSION

The senescence of carnation petals is associated with increased production of the phytohormone ethylene, which plays a role in the initiation and coordination of senescence events (2, 15, 16). We have shown previously that petal senescence is associated with the appearance of several new mRNAs and that many of these mRNAs can be induced in presenescent petals by exposure to exogenous ethylene (25, 26). To understand the relationship between ethylene, gene expression, and petal senescence, we have isolated and characterized a number of senescence-related cDNA clones that were used to study specific changes in gene expression during the course of this developmental process.

The three cDNA clones that have been characterized hybridize to individual mRNAs that increase in abundance as the flower petals senesce. These mRNAs also accumulate after exposure of flowers to ethylene, suggesting that ethylene plays a role in the expression of these genes, as it does in the overall development of senescence. Inhibitors of ethylene biosynthesis and action virtually eliminate the accumulation of the mRNAs that hybridize to pSR8 and pSR12, whereas the mRNA detected by pSR5 is only somewhat reduced by these treatments. These results can therefore be interpreted as indicating that at least two classes of mRNAs are expressed at higher levels during senescence of carnation petals. The first class, represented by pSR5, is regulated by both ethylene and temporal factors, whereas the second class, identified by pSR8 and pSR12, is strictly controlled by ethylene. It is likely that these exemplify some of the mechanisms operating to regulate gene expression during flower senescence.

Lincoln *et al.* (11, 12) have shown that gene expression in tomato fruits is regulated by changes in both the concentration of, and sensitivity to, ethylene during the course of ripening. We have reported (26) that aging of carnation petals is accompanied by an increase in the ability of ethylene to induce

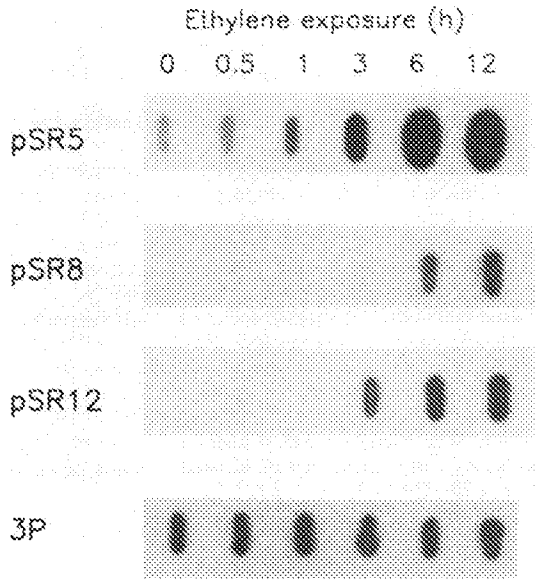

Figure 6. Accumulation of specific mRNAs in carnation flower petals exposed to 7.5 μL/L ethylene for various durations. Flowers were harvested and exposed to ethylene as previously described (26). Immediately following removal from the ethylene atmosphere, petals were frozen in liquid N_2 and total RNA isolated. Ten μg of total RNA was slot-blotted onto nitrocellulose and hybridized to ^{32}P-labeled cDNA clones. Blots were exposed to x-ray film with an intensifying at −70°C screen for 18 h.

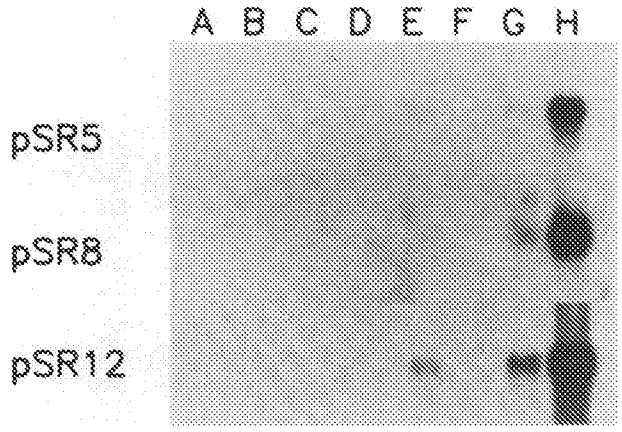

Figure 7. RNA-blot analysis of RNA isolated from various vegetative and floral organs of carnation. Ten μg of total RNA isolated from various tissues was separated electrophoretically under denaturing conditions on 1.2% agarose, blotted to nitrocellulose, and hybridized to ^{32}P-labeled cDNA clones. Lane A, recently matured leaves; B, stems; C, roots; D, preclimacteric flower calyx; E, climacteric flower calyx; F, preclimacteric flower gynoecia; G, climacteric flower gynoecia; and H, climacteric flower petals. RNA blots hybridized to pSR5, pSR8, and pSR12 were exposed to x-ray film with an intensifying screen at −70°C for 18, 36, or 18 h, respectively.

autocatalytic ethylene biosynthesis, petal senescence, and changes in mRNA populations. How this process of development influences the expression of senescence-related genes will be the focus of further research.

The pleiotropic effects of ethylene on the growth and development of plants may be the result of differential gene expression. Ethylene has been shown to regulate the transcription of genes in both tomato fruits (11) and carrot roots (19). In climacteric fruits, prolonged exposure to ethylene (24 h) was required to induce the expression of several genes involved in fruit ripening (21). The results presented here indicate that ethylene is able to modulate the expression of senescence-related genes over a much shorter period of time (1–3 h). A similar rapid induction of ethylene regulated genes has been reported in tomato fruits (12). The accumulation of senescence-related mRNAs in presenescent flowers that have been exposed to ethylene occurred before the development of any visible symptoms of senescence. This is consistent with the hypothesis that changes in petal physiology in response to ethylene are the result of rapid changes in gene expression. The perception of, and response to, ethylene are strictly required for carnation petal senescence to occur (2). Ethylene has been demonstrated to be involved in the senescence of many plant organs. Elucidation of how these processes are controlled will contribute to our understanding of senescence in plants.

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